Parnassus Flow Cytometry CoLab

FACSAriaII SuperUser Training Guide (STAFF/glossary)

Concepts and materials relevant to flow core staff, used to aid training of unassisted user setup

Startup procedures

To be completed if first user of the day. User needs to turn on instrument/cooler and check waste is empty and sheath is full to weld line. Talk about the pressure valve on the sheath tank, always pull up and turn right to seal the valve so it doesn't loosen over time. Go over sensors (waste, cart plugin), and how they can trigger errors in software. Check sheath filter and de-bubble if necessary. Explain the need to clean up spills / corrosive salts and push the cart back in to maintain stable fluidics when finished. Insert closed loop nozzle to send spent volume to the waste during Fluidics Startup to prevent splashing, unwanted salt build-up, and stress on nozzles. While Fluidics Startup runs, have them make cleaning tubes, fill the waste drawer with ethanol (make sure it aspirates evenly near the end of Startup), and clean the sort block / voltage plates (show how to use the plate removal tool). Emphasize how dried salts, especially on plates, can interfere with sort. Listen for pressure leaks and explain how to check and tighten the tank lid.

Starting the stream

Once Fluidics Startup and general cleaning is finished, have them select their nozzle (confirm M/MS), make sure it (and the nozzle seat) is free of salts, dry, and red O-ring is present. Show how to insert nozzle carefully to avoid scraping or losing the O-ring. Show user where nozzle backups are. If the O-ring is lost before experiment, a replacement may be used. If the O-ring is lost mid-experiment, a replacement nozzle may be used, **BUT** Automatic CST must be run again before continuing**.** Explain different configurations are used for each nozzle because they set the instrument at the correct pressure for the specific nozzle aperture. Go over stable stream characteristics by eye (stream is centered in waste slot, isn't fanning) and in software image view (straight, drop formation, satellites) and when they might need to wiggle the position or check nozzle aperture under microscope and sonicate if obstructed. If the stream won't stabilize, check for bubbles in the sheath filter, or an issue with the sheath line (caught under the cart, etc.). Show how to use applicator in sort block for streaked stream window. Explain any extra steps for UV laser startup.

Manual CST QC

CST beads include 2 sizes and 3 fluorescence intensities (dim, medium, high). Teach them to look for the 3 peaks corresponding to the intensities of the beads, making sure they are relatively thin and sharp, with clean separation. This ensures the lasers have adequate power, the instrument is clean, and that we are able to capture optimal resolution and sensitivity. If peaks are very wide or overlapping, the flow cell may need cleaning. If the peak(s) are missing from one channel for one laser, show them how to physically check the configuration of filters inside the instrument. If the peak(s) are absent from every channel for one laser, that laser delay may be wrong, or the laser may be dying. In this case, have them move on to Automatic CST QC to recalculate the optimal delay, **BUT** they must re-run Manual CST afterward to confirm the peak(s) have become visible.

Automatic CST QC

Explain the automated program is a performance check used to track consistency and generate / update default cytometer settings of the Laser delays and place each PMT within an optimal range to distinguish dim, medium, and bright emissions of the test fluorochromes. It compares current performance with a baseline previously set for the specific lot number of the CST beads and calculates any changes in laser delays and area scaling. Explain how to proceed with warnings or failures:

If it **passes** with no warnings, it is okay to finish and move on.
If it **passes** with a warning on one or two PMTs (change in voltage relative to baseline), it is still usable.
If it **passes** with a warning on robust CVs, double check the manual peaks, and if they are very wide → clean flow cell.
If it **fails** on **Δ**PMTV (a large shift in voltage), it usually has to do with FSC and may need cleaning.
If it **fails** on **Δ**PMTV for one PMT, it is possible the filter was swapped out.
If it **fails** on all PMTs for the same laser, it could be a dirty component or dying laser. Make any necessary changes & repeat CST.

Before proceeding with a failed run, take care that the laser delays have not been reverted to 0.00.

Stable Stream

Explain the importance of establishing a stable stream before setting the drop delay in order to maintain accurate timing of stream charging when the target is at the breakoff point. Typically, when setting up sorting, use the Amplitude to set the required drop breakoff at the correct Gap value and update the generated Drop 1 with the actual value (on the right) to the target field (on the left). The best way to make sure a stream is truly stable after setting values is to watch it for a few moments with Sweet Spot off; the breakoff point should not be drifting. Then, turn on the Sweet Spot to autopilot the amplitude and maintain the breakoff point. Explain what test sort streams should look like (tight, round center stream and visible sort streams all in focus) and how to refine them with the micrometer and Drop 2 to Drop 4 values.

Stream Glossary

Sweet Spot: enables automatic adjustment of the drop drive amplitude to maintain stability of drop breakoff point.
When the Sweet Spot detects a >1-pixel difference between the target Gap setting and the actual Gap, it adjusts the Amplitude to match the gap. When a >2-pixel difference is detected, the Sweet Spot stops sorting (charging the stream) temporarily until the Gap is restored, although the sample continues to flow. Once restored, sorting automatically resumes.

Amplitude: adjusts the amplitude or intensity of the acoustic energy driving drop formation. A charge from 1-80 volts is applied to a piezoelectric crystal touching the nozzle and determines the breakoff point of the stream. Higher amplitude results in shorter stream breakoff. Amplitude is usually set once and maintained via Sweet Spot (unless stream becomes unstable, avoid changing the amplitude mid experiment as it may alter breakoff point/ reduce accuracy of the drop delay setting).

Frequency (don't touch!): determines the number of drops formed per second, and the size of the drops (also influenced by nozzle size). Frequency can be set from 1-102 kHz. The higher the frequency, the more drops generated per second and the smaller the drops (**defaults: 100um/30kHz, 70um/90kHz, 85um/45-50kHz, 130um/12-15kHz**).

Drop 1: the distance between the top of the stream image and the center of the first broken-off drop, from 100 to 600 pixels (acceptable variation of 10 pixels between target and actual).

Gap (don't usually need to change): the gap between the stream breakoff and the top of the first detached drop, in pixels (**defaults: 100um/ 10 pixels, 70um/ 7 pixels, 85um/8 pixels, 130um/13 pixels**).

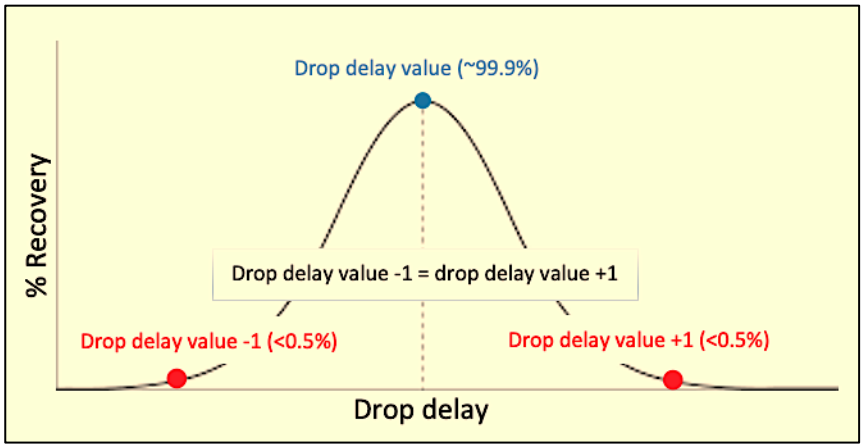
Satellite drops: smaller drops between actual drops, generated by drop drive right after breakoff. The behavior of these small drops helps determine stable breakoff. Satellite merging is largely dependent on nozzle position. If not merging, wiggle sideways, remove and reinstall nozzle, or even clean it by sonicating.

Attenuation: decreases the amplitude of the drop drive. At lower pressures (e.g. 100um), you may need to turn on attenuation to dampen the amplitude to further stabilize the stream.

Drop 2, 3, 4: once drop 1 is charged and breaks off, a neutralizing charge needs to be applied to the stream to prevent subsequent drops 2,3,4 from also being charged and deflected. Drop 2, 3, 4 values are a correction factor for the drop charge as a percentage of the previous drop, from -100 to 100%. Generally, these settings shouldn't vary drastically from the prior day, and the drop 2 value should not be below 10, otherwise there may be voltage issues. The values for subsequent drops usually decrease by about half; for example, the values may be set at drop 2= 16, drop 3= 8, and drop 4= 4.

Accudrop QC

This is how we calculate a dependable (drop) charge delay, and which drop contains the particle of interest that should be charged to sort. The drop delay is the amount of time between when an event is measured at laser interrogation point and the breakoff point, from 10-140 drops. The drop delay value determines when the stream is charged, so the next drop to break off is charged and deflected (sorted). It is set experimentally using BD FACS Accudrop technology. In theory, this drop delay setting is based on sort efficiency of Accudrop beads on a normal curve. The correct drop delay should be at the top of this curve, close to 99.9% efficiency. After finding this value by changing the value in the drop delay field, confirm it by checking the efficiencies at the value +/- 1 drop. These values should be identical to each other and >95% if the drop delay setting is accurate. Don't forget to change the value back to the actual drop delay after this check!



Tube calibration

Explain the stream needs to be hitting media in the bottom of the collection vessel, and that deflecting cells into the tube wall can be lethal for cells (coating tube walls with media helps). Remind the user it often causes issues for next users to close any sorting streams, even if they may not be in use (e.g., they only need the inner tubes of the 1.7 mL collection device). Clarify keeping those streams open will **not** be an issue for a sort, since there is no population being sorted there. DO NOT CLOSE SORT STREAMS TO ZERO ON 4-TUBE COLLECTION DEVICES.

Suggested Learning Experiences (for testing users)

Rotate and set up on multiple instruments (each has unique common issues)
Opened pressure valve in Fluidics Startup
Unplug fluidics cart
Unplug waste sensor
Substitute a broken nozzle
Throw off frequency
Shake sheath line
Throw off micrometer
Change precision mode on Accudrop Experiment template (from Fine Tune)

To demonstrate dependence of the Drop Delay on Drop 1, after optimal Drop Delay has been determined, change Drop 1 to demonstrate how the Drop Delay no longer works.

Another demonstration, lightly shake the sheath line to show how easy it is to create a very unstable stream. After this, emphasize that when the Sweet Spot is on and something like this happens, sorting will automatically pause, saving the purity of the sort (although the sample will still be running). When Sweet Spot is off, sorting is never paused, so an unstable stream for any amount of time compromises purity. **Use Sweet Spot!**